CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL FROM *CYMBOPOGON CITRATUS* AND *CYMBOPOGON NARDUS*

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ABSTRACT

The bacterial resistance has created a major health issue worldwide whereby the pathogens becoming resistant even to the most recently approved antibiotics. Essential oils have showed many biological activities such as antibacterial, antifungal, antiviral, antioxidant and insecticidal. This study was conducted to analyse the chemical composition of the essential oils of *Cymbopogon citratus* and *Cymbopogon nardus*; and to study their antibacterial activities in alone and in combination. Essential oils obtained by steam distillation were analysed by gas chromatography-mass spectrometry (GC-MS); while the antibacterial activity of the essential oils were evaluated against five bacteria namely *Enterococcus faecalis* ATCC 14506, *Staphylococcus aureus* BAA-1026, *Bacillus Subtilis* ATCC 11774, *Escherichia coli* ATCC 10536, and *Salmonella typhimurium* ATCC 14506 by using disk diffusion and broth microdilution methods. To determine the antibacterial effects of essential oils in combination, the broth microdilution checkerboard method was utilized. From the results, it is observed that the major compounds contained in essential oils of *Cymbopogon citratus*, and *Cymbopogon nardus* were geranial (33.01%) and elemol (44.14%), respectively. The result of antibacterial activity indicated that *Cymbopogon citratus* possessed a good and wide spectrum of antibacterial activity against all the tested bacteria; whereas *Cymbopogon nardus* only showed stronger antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. Gram-positive bacteria were more sensitive to the investigated oils than Gram-negative bacteria; in which *Staphylococcus aureus* was the most sensitive strain tested, with the lowest MIC value (0.47µl/ml). The *Cymbopogon nardus* had showed greater bactericidal activity against all Gram-positive bacteria compared to *Cymbopogon citratus*. The result of antibacterial activity of essential oils in combination showed that the combination were less effective compared to when each of the essential oils was used individually; the antagonism responses were obtained against all the tested bacteria except for *Enterococcus faecalis* bacteria which showed indifference response. The results presented may suggest that the essential oils of *Cymbopogon citratus* and *Cymbopogon nardus* could be employed as a potential source of antibacterial ingredients for food and pharmaceutical industry; however, it is recommended for not mixing these both essential oils as they have not given positive results for antibacterial activity.

ABSTRAK

Kerintangan bakteria telah mewujudkan satu masalah kesihatan utama di seluruh dunia di mana patogen menjadi kebal walaupun antibiotik yang baru ditemui. Minyak pati telah menunjukkan pelbagai aktiviti biologi seperti antibakteria, antikulat, anti-virus, anti-oksida dan anti-serangga. Kajian ini dijalankan untuk menganalisis komposisi kimia minyak pati *Cymbopogon citratus* dan *Cymbopogon nardus*, dan untuk mengkaji aktiviti antibakteria mereka secara bersendirian dan kombinasi. Minyak pati yang diperolehi daripada penyulingan stim dianalisis oleh kromatografi gas- spektrometri jisim (GC-MS), manakala aktiviti antibakteria telah dinilai terhadap lima jenis bakteria iaitu *Enterococcus faecalis* ATCC 14506, *Staphylococcus aureus* BAA - 1026 , *Bacillus subtilis* ATCC 11774 , *Escherichia coli* ATCC 10536 , dan *Salmonella typhimurium* ATCC 14506 dengan menggunakan kaedah penyebaran cakera dan kaedah kaldu mikrocairan. Untuk menentukan kesan antibakteria minyak pati dalam gabungan, kaedah kaldu mikrocairan dam telah digunakan. Keputusan yang diperolehi menunjukkan bahawa sebatian utama yang terkandung di dalam *Cymbopogon citratus* dan *Cymbopogon nardus* adalah geranial (33.01%) dan elemol (44.14%), masing-masing. Hasil keputusan daripada ujian aktiviti antibakteria pula menunjukkan bahawa *Cymbopogon citratus* memberikan spektrum yang baik dan meluas terhadap semua bakteria yang diuji; manakala *Cymbopogon nardus* hanya menunjukkan aktiviti antibakteria yang kuat terhadap bakteria Gram positif daripada bakteria Gram-negatif. Bakteria gram-positif adalah lebih sensitif kepada minyak pati yang diuji daripada bakteria Gram-negatif, di mana *Staphylococcus aureus* merupakan bakteria yang paling sensitif, dengan nilai MIC terendah, 0.47µl/ml. *Cymbopogon nardus* juga telah menunjukkan aktiviti bakteria lebih berkesan terhadap semua bakteria Gram-positif berbanding Cymbopogon *citratus*. Hasil daripada aktiviti antibakteria minyak pati dalam gabungan menunjukkan bahawa kombinasi kurang berkesan berbanding apabila setiap minyak pati digunakan secara sendirian; tindak balas antagonistik telah diperolehi terhadap semua bakteria yang diuji kecuali kepada *Enterococcus faecalis* yang menunjukkan tindak balas sebaliknya. Kajian ini mencadangkan bahawa minyak pati *Cymbopogon citratus* dan *Cymbopogon nardus* boleh digunakan sebagai salah satu sumber bahan antibakteria dalam industri makanan dan farmaseutikal. Walau bagaimanapun, ia tidak digalakkan untuk mencampurkan kedua-dua minyak pati ini untuk aktiviti antibakteria memandangkan keputusan yang diperolehi adalah kurang berkesan.

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LIST OF SYMBOLS

%	Percent
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
µl	Microliter
µl/ml	Microliter per mililiter
µm	Micrometer
cfu/ml	Colony forming unit per mililiter
eV	Electron volt
g	Gram
h	Hour
m	Meter
M	Molarity
m/z	Mass to charge ratio
mg/ml	Miligram per milimeter
min	Minutes
ml	Mililiter
ml/min	mililiter per minute
mm	Milimeter
nm	Nano meter
ºC	Degree celcius
pH	A Measure of Acidity or Basicity

rpm	rotation per minute
s	Second
v/v	Volume per volume
w/w	Weight per weight
α	Alpha
β	Beta
γ	Gamma

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BC	Before Christ
C. citratus	*Cymbopogon citratus*
C. nardus	*Cymbopogon nardus*
CLSI	Clinical and Laboratory Standards Institute
-CoA	-coenzyme A
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethylsulfoxide
EOs	Essential Oils
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Indices
FPP	Farnesyl pyrophosphate
GC-MS	Gas Chromatography-Mass Spectrometry
GPP	Geranyl pyrophosphate
IPP	Isopentenyl pyrophosphate
KDO	Ketodeoxyoctonate
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MHB	Mueller Hinton broth

MIC	Minimum Inhibitory Concentration
MVA	Mevalonic acid
OD	Optical density
PP	Pyrophosphate
RM	Ringgit Malaysia
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Over the last 50 years, there is a growing study on plant secondary metabolites (Bourgaud et al., 2001). These plant secondary metabolites are known to contribute a major role in the adaptation of plants to their ecological interactions. For instance, they could act as protector against herbivory and microbial infection (which also been described as antibiotic, antifungal, and antiviral), as attractants for pollinators and seed dispersing animals, and as allelopathic agents (allelochemicals that influence competitions among plant species). In addition, they also contain important UV absorbing compounds to prevent serious leaf damage from the light (Bourgaud et al., 2001; Croteau et al., 2000 and Hyldgaard et al., 2012).

The plant secondary compounds consist of three major groups: phenolics, terpenes and steroids, and alkaloids. These groups are classified according to their biosynthetic pathways; phenolics and alkaloids are derived from shikimic acid pathway while terpenes and steroids from acetyl-CoA mevalonic acid pathway (Croteau et al., 2000; Bourgaud et al., 2001 and Ramawat et al., 2009).

Many biological activities showed by plant secondary metabolites have long been used in traditional medicine (Bourgaud et al., 2001). Traditional medicine is defined as the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigeneous to different cultures used in the health maintenance,

prevention of diseases and improvement of physical and mental diseases (Ramawat et al., 2009). One of the types of traditional medicine is herbal medicine; it is also known as medicinal plants (Effendy et al., 2012).

Malaysia is gifted with a wide variety of herbal medicine and these medicine have served as the primary healthcare for locals since ages (Mustaffa et al., 2011 and Effendy et al., 2012). From a global survey report by WHO, it shows that Malaysia was one of the nine countries that contributed a large amount of sales in herbal medicine worldwide between the year of 1999 to 2001 (Figure 1.1). In 2008, the Malaysian market for herbal and natural products was estimated to growth approximately RM10 billion with the raise of 8% rate per year (Effendy et al., 2012). At the same time, referring to World Bank report, they predicted that during 2050, the global market for herbal products would be about 5 US trillion dollars (Rasadah and Ali, 2008).

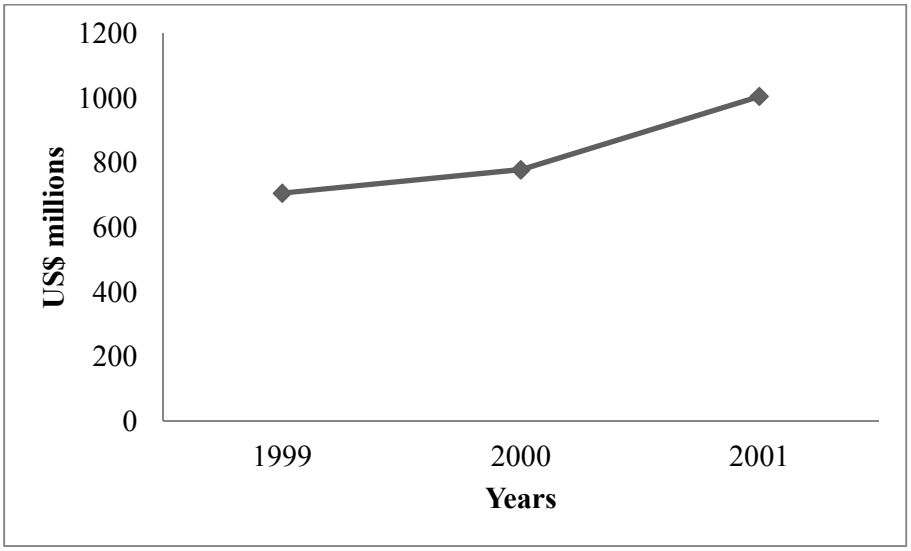

Figure 1.1: Growth in the sales of herbal medicine of nine representative countries from 1999-2001 (Bhutan, Canada, the Czech Republic, Iran, Madagascar, Malaysia, Pakistan, Sudan and Sweden)

Adapted from: Effendy et al. (2012)

In relation to this, the government urged researchers, academicians and industry operators to grab the opportunity by speeding up their research and development activities in medicinal plants to find new leads and could market them worldwide (Rasadah and Ali, 2008).

1.2 PROBLEM STATEMENT

The extensive use of antibiotics in human medicine, in animal production and as growth promoters in agriculture has led to the increase of bacterial resistance (Palaniappan and Holley, 2010). This bacterial resistance has created a major health issue worldwide whereby the pathogens becoming resistant even to the most recently approved antibiotics (Figure 1.2) (Huh and Kwon, 2011). These resistant organisms may be transferred to humans in two ways; either directly via the food chain or indirectly as a result of spread of animal waste in fields (Palaniappan and Holley, 2010).

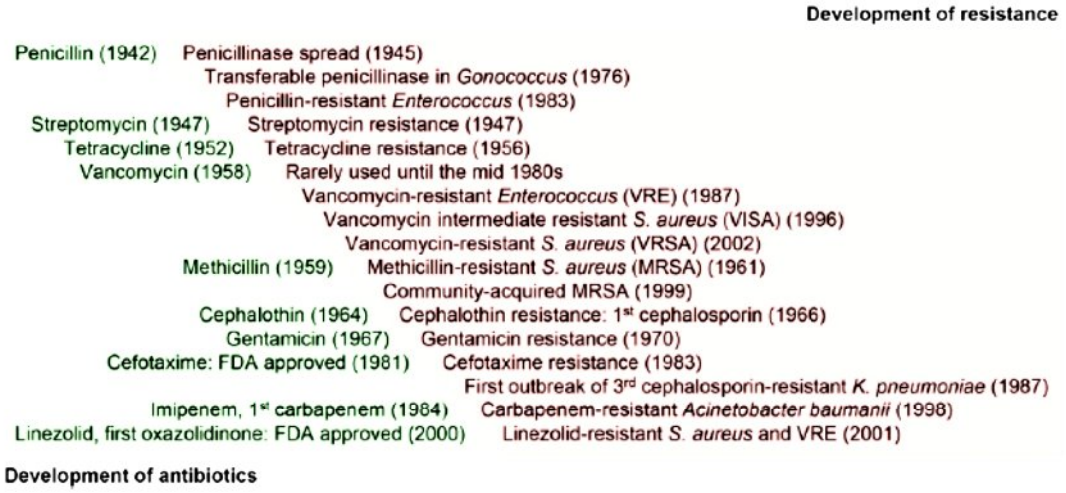

Figure 1.2: History of antimicrobial agent development vs. subsequent acquaintance of resistance by microorganisms

Source: Huh and Kwon (2011)

Due to the increasing dilemma of antibiotic resistance, adverse effects and high costing have led researchers to explore natural resources especially plant materials as an alternative source of antimicrobials (Zaidi et al., 2009). In relation to this, the study about essential oils from various plants in Malaysia has been done extensively by the

researchers to discover their beneficial potential. Many of the essential oils from the plants have shown their potential as antimicrobials (Hossain et al., 2011; Ibrahim et al., 2009; Maizura et a., 2008). However, as far as the articles could be ascertained, there is no yet study about antibacterial activity of essential oils in combination from Malaysian medicinal plants. Therefore, this study will highlight the antibacterial activity of essential oils from Malaysian medicinal plants, *Cymbopogon citratus* and *Cymbopogon nardus* independently and in combination.

1.3 OBJECTIVES OF THE RESEARCH

The objectives of this study are:

i. To analyse the chemical composition of *Cymbopogon citratus* and *Cymbopogon nardus* essential oils by using Gas Chromatography-Mass Spectrometry (GC-MS).
ii. To study the antibacterial activity of the essential oils *Cymbopogon citratus* and *Cymbopogon nardus*
iii. To study the antibacterial activity of the essential oils of *Cymbopogon citratus* and *Cymbopogon nardus* in combination through broth microdilution checkerboard assay.

1.4 SCOPES AND LIMITATION OF THE STUDY

This study focuses on the screening for antibacterial agents of essential oils from the Malaysian medicinal plants, *Cymbopogon citratus* and *Cymbopogon nardus*. These species are well known in Malaysia and have been used by Malaysian herbal manufacturers to produce a wide variety of health related products. This study is limited to the chemical identification and antibacterial activity from the medicinal plants.

1.5 SIGNIFICANCE OF THE STUDY

The results of this study would contribute a new, inexpensive and alternative antibacterial agent from Malaysian medicinal plants. In addition, it is hoped that this research will help the medicinal plant research and development to gain an insight into

the effectiveness of our local herbal traditional formulations as most of them are prepared in combination of more than one ingredient.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORICAL REVIEW ON AROMATIC SUBSTANCES AND EOs

The study of essential oil (EO) is a long history; it was started since antiquity until this modern world. This study is never ending; every time there are new findings are discovered. The researchers work very hard in order to maximise the use of EOs (Surburg and Panten, 2006).

2.1.1 The use of EO in ancient time

It is known that the aromatic sources which originated from spices, resins from animals and plants have been used enormously since ancient times for perfumery, flavour purposes and in health care system (Buckle, 2003 and Surburg and Panten, 2006). Dates back to the fourth century Before Christ (BC), Hippocrates, the well-known father of medicine in Greek employed the burning of aromatic substances to prevent from contagious diseases; he also suggested the Greeks and Romans to add aromatic oils in their bath houses for their health (Worwood, 1991).

In another reports, it was stated that the Egyptians has written the oldest documentation of therapeutic treatments and pharmaceutical plant preparations namely 'Papyrus Ebers' in 1500 BC. Correspondingly, the aromatic substances were discovered in Tutankhamen's tomb; this showed that their priests had used aromatic substances to embalm the pharaoh's body from decaying. The Babylonians are also one of the earliest people who used those sources in their daily lives. They favoured to use oils of myrrh

(*Commiphora* spp.), frankincense (*Boswellia* spp.) and cedarwood to treat various diseases (Marshall, 2004 and Worwood, 1991).

Instead of those peoples, the Arabs, Indians, and Chinese also have been reported to use the aromatic subtances. For instance, in China, it was reported that the first text of procedure on herbal medicine preparation was found around 2800 BC. To treat the transdermal illness, the Chinese absorbed a cloth in herbs and put it on the skin because they believed that the benefits contained in the herbs may permeable through the skin (Buckle, 2003 and Worwood, 1991).

In India, the Ayurvedic medicine had been practised approximately in 2000 BC; this was found in their first Sanskrit medical treatises, *Caraka Samhita* and *Sushrata Sambita*. The manuscripts described the use of 700 plants and many of them are aromatics such as ginger, coriander, myrrh, cinnamon and sandalwood (Buckle, 2003).

In Arabia, the Arabs had improved the use of herbal and aromatic medicine by introducing new aromatics such as senna, camphor, tamarind, nutmeg, and cloves to the list of medicinal plants. The Arabs also recommended to add in the rose and orange-blossom water in giving the anaesthetic effect (Buckle, 2003). The famous medical textbook, *Canon of Medicine* written by Ibn Sina or Avicenna was translated from Arabic to Latin and had spread to Europe in the twelfth century. This *Canon* lists 760 medicinal plants and the drugs that can be derived from them. This knowledge has led Europe to apply it in treating the disease caused by bad odours by using aromatics waters like “eau de cologne” (Buckle, 2003 and Worwood, 1991).

Consequently, the importance of aromatic natural products has resulted in the discovery of the technique for its preparation. Hence, the distillation technique to obtain EOs has been introduced in 9^{th} century A. D. and it was reported that the person who is responsible to this was Ibn Sina. He called the distillation apparatus as *alembic* (Buckle, 2003; Burt, 2004 and Surburg and Panten, 2006).

2.1.2 The EOs in Europe

The use of EOs in aromatherapy is very well known in a part of country such as in United Kingdom, United States and France. In the United Kingdom, the EOs are commonly been applied in the massage to reduce stress and in other health care system; while in France, the EOs are diluted in vegetable oil and be given orally in a gelatin capsule by a medical or herbal doctor. This oral application is effective to treat gastrointestinal problem and to fight an acute or chronic infection (Buckle, 2003).

In the United Kingdom, the effort to evaluate the EOs scientifically has been started in the nineteenth century and many of these results have been recorded in *Materia Medica and Therapeutics* (1882) published by William Whitla. As the idea to identify and isolate therapeutic components of the plants become crucial later, in the late 1890s the specific components in essential oil such as geraniol and citronellol have been successfully identified (Buckle, 2003).

In France, the efforts to use EOs in the health care and disease treatments have been introduced by the first pioneers of modern aromatherapy: Gattefosse (a chemist), Valnet (an army physician), and Maury (a nurse) (Buckle, 2003).

Rene-Maurice Gattefosse was the person who had introduced the word *aromatherapy*. He was very interested in the research of topical application of EOs after he accidentally used one rinse of essential oil of lavender *(Lavandula augustifolia)* to treat the wounds that infected with gas gangrene when he was injured in a fire. Surprisingly, the wounds that had been treated with the essential oil had healed. This incident has brought him to do more research on EOs all his life (Buckle, 2003 and Worwood, 1991).

His research became beneficial when the EOs of thyme, chamomile, clove, and lemon were used on infected wounds, gangrene treatment and as sterilizer for surgical instruments in World War I and World War II (Buckle, 2003).

On the other hand, Jean Walnet had spent much of his life researching aromatherapy and he really believed the powerful of EOs to keep away from accidents and incidents. He also had applied the use of EOs when he served as a commander of an

advanced surgical when he was in Indochina. He had written a book of classic aromatherapy entitled *The Practice of Aromatherapy* and it has been translated into many languages such as English, German, Italian, Spanish, and Japanese (Buckle, 2003).

Marguerite Maury has given her contribution to the public by categorizing the use of EOs into various clinical departments: surgery, radiology, dermatology, gynecology, general medicine, psychiatry, spa treatment, physiotherapy, sports and cosmetics. Her efforts paid off when she had won two international prizes in the research of EOs and dermatology; and her book, *Le Capital Jeunesse* has been translated into English (Buckle, 2003).

2.1.3 The EOs in Malaysia

Malaysia, which is located in the Southeast Asia and on the equator, only facing with hot and humid throughout the year. This region, which also surrounded with oceans receives rainfall about 200 centimetres (79 inches) and the temperatures varying from 20ºC to 35 ºC (70º to 100ºFahrenheit) each year. This equatorial climate has categorised Malaysian forests as tropical rainforests (Bodeker, G. et al., 2009).

The rainforests is invaluable gift to Malaysia as it contains with an extremely rich biodiversity. Due to its extent of the biological diversity, Malaysia has been recognized as one of 12 global mega diversity areas in the world (Syukor, A.R.A. et al., 2008). Regarding to this, the Malaysian people are very fortunate because this rainforests sources are very close at their hand. Plants can be picked, mashed, cooked, consumed and applied at all times of the year (Bodeker, G. et al., 2009).

In particular, they used this source in their traditions to improve their health and beauty. The term *ramuan* is used in Malay language which refers to a healing mixture of medicinal plant and plants part. This *ramuan* is considered as a force of healing, beauty and vitality. Another terms that are also commonly used by the villages are: *rempah ratus* (a term refers to a polyherbal preparation from a hundred kinds of medicinal plants and spices), *ramuan akar kayu* (plant roots mixture) and *ramuan asli* (original plants mixture). This *ramuan* was inherited from generation to generation of their ancestors (Bodeker, G. et al., 2009).

Generally, they used this *ramuan* in their daily lives; for example, for facial and skin care, in traditional dental care, in bridal grooming, in pre-natal and post-natal care, for nursing mothers, and in traditional herbals for male vitality. Commonly, they used the *ramuan* preparation in form of herbal masks and scrubs, flower baths, scented steams and herbal oils (Bodeker, G. et al., 2009).

There are many plants used by Malaysian in their practices, such as: mashed noni fruit (*Morinda citrifolia*), oil of coconut milk (*Cocos nucifera*), keremak leaves (*Alternanthera sessilis* L.), buah keras (*Aleurites moluccana*) and pandan leaves (*Pandanus odorous*) have been used in the hair care; while a warm herbal bath consisted of sweet lemongrass (*Cymbopogon nardus),* betel leaves (Piper betle), pandan leaves and slices of ginger, *asam keping* (*Garcinia atroviridis*) were used in women personal hygiene. Other than that, for postpartum remedies, the specialist herbs which are commonly used by Malays are *Kacip fatimah* (*Labisia pumila*), mas cotek (*Ficus deltoidea*) senduduk (*Melastoma malabathricum*) and many more (Bodeker, G. et al., 2009 and Jamal et al., 2011).

However, from the reviews, it can be observed that the use of essential oils from the plants in Malaysia is not too familiar among the old folks; it only could be found widely in this modern era after scientific studies are beginning to validate the efficacy of some of these traditional formulations and the country is becoming more aware of the therapeutic and commercial potential of the *ramuan* tradition. Hence, nowadays, the researchers in Malaysia are very exciting to find new scents and properties of essential oils from the Malaysian rainforest plants it is always on growing (Bodeker, G. et al., 2009).

2.2 SYSTEMATIC INVESTIGATIONS OF CONSTITUENTS FROM EOs

By the 13th century, the pharmacies started to produce the EOs and describe their pharmacological effects in pharmacopoeias. This effort was the beginning to the systematic development of the EOs (Surburg and Panten, 2006). However, the first systematic investigation of constituents from essential has been performed by M. J. Dumas, a French chemist. He analysed some hydrocarbons, oxygen, and sulphur- and nitrogen-containing constituents (Kubeczka, 2010). In 1834, he and PELIGOT had

isolated cinnamaldehyde from cinnamon oil and followed by the isolation of benzyldehyde from bitter almond oil by LIEBIG and WOHLER in 1837 (Surburg and Panten, 2006).

Later, a new part of the chemical industry in the history of natural fragrance materials was opened when the fragrance and flavour chemicals could be produced synthetically and industrially. This shift began with the production of acid esters of several alcohols (in 1845 and 1850), followed by methyl salicylate (1859), benzaldehyde (1870), vanillin (1874) and coumarin (1878) (Surburg and Panten, 2006)

In particular, the study about EOs has brought to a number of scientists be honoured for Nobel Prize. It was began with Otto Wallach (German chemist) who was honoured for Nobel Prize in Chemistry "in recognition of his outstanding research in organic chemistry and especially in the field of alicyclic compounds" in 1910 (Surburg and Panten, 2006). He has dedicated his life to study about terpenes, which enormously found as the major constituents in EOs. His book, *Terpene und Campher* was a compilation of his 180 articles and the knowledge on terpenes (Kubeczka, 2010). His efforts have resulted in the most important finding in the study of terpenes, which is the discovery of isoprene rule. This rule explained that the terpene compounds were constructed from isoprene unit (C_5H_8) which joined together in a repetitive head-to-tail manner (Carson and Hammer, 2011 and Kubeczka, 2010).

Instead of Wallach, Leopold Ruzicka also was awarded the Nobel Prize in Chemistry (1939) for his outstanding investigations in structure elucidation for his work on "polymethylenes and higher terpenes". This followed by D.H.R Barton (English chemist) who was awarded the Nobel Prize in Chemistry in1969 for his discovery on the structure of caryophyllene which has a 4- and 9-membered ring (Carson and Hammer, 2011 and Kubeczka, 2010).

Other chemists who also showed their contributions were F.W Semmler and G. Wagner (1899); they discovered about rearrangement for the elucidation of chemical constitution on some of acyclic monoterpenes like geraniol, linalool, and citral. This investigation was continued by H. Meerwein (1914) and later he generalized it as Wagner-Meerwein rearrangement. Furthermore, J. Read, W. Huckel, H. Schmidt, W. Treibs, and V. Prelog had explained the stereochemical structures that occur on